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# The effect of two different Individually Ventilated Cage systems on anxiety-related behaviour and welfare in two strains of laboratory mouse

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## HIGHLIGHTS

- We studied the impact of two IVC housing systems on behaviour and welfare in mice
- We found differences in anxiety-related behaviour between the two housing systems
- Different IVC housing systems appear to affect mouse behaviour in different ways

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## ABSTRACT

The environment in which a laboratory animal is housed can significantly influence its behaviour and welfare, acting as a potential confounding factor for those studies in which it is utilised. This study investigated the impact of two Individually Ventilated Cage (IVC) housing systems on anxiety-related behaviour and welfare indicators in two common strains of laboratory mice. Subjects were juvenile female C57BL/6J and BALB/c mice (N = 128) housed in groups of four in two different IVC systems for 7 weeks. System One had air delivery at the cage 'cover' level at 75 ACH (Air Changes/Hour) and System Two had air delivery at the 'animal' level at 50 ACH. Mice were assessed twice a week (e.g. bodyweight) or at the end of the study (e.g. anxiety tests). Our results showed significant differences in anxiety-related behaviour between strains and housing systems. Mice in System Two, regardless of strain, defecated more in the Elevated Plus Maze (EPM), spent less time in the open arms of the EPM, and less time in the central zone of the Open Field (OF). Strain differences in anxiety-like behaviour were seen in the increased defecation by BALB/c mice in the OF and EPM and less time spent in the open arms of the EPM compared to C57BL/6J mice. These results suggest that different IVC housing systems can influ-



## 1. Introduction

The housing environment of laboratory animals has been demonstrated to have a significant influence on the behaviour, physiology, pathology and brain development of the animals housed within [1–5], thereby potentially impacting upon the comparability and reproducibility of the data generated [6,7]. It is therefore important to identify what impact different aspects of the housing environment (e.g. stocking density, internal cage complexity) can have in order to be able to maximise both the welfare of the animals used in scientific studies (in accordance to European and International legislation) and ensure scientific rigour. Such an approach also allows modulating environmental factors, once identified, to be taken into consideration during experimental design and subsequent statistical analysis [8].

One important aspect of the housing environment is the design of the cage itself, and the manner in which it is managed. Traditionally, 'open' (conventional) cages have been used that are ventilated by the room ventilation system in which they are located. Open cages risk exposure of the animals to microorganisms present in the room and an increased

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exposure to potential allergens for human workers within the same environment. For this reason, and to maintain adequate ventilation, low relative humidity and reduced concentrations of ammonia and $CO_2$ [9,10] in the cages, Individually Ventilated Cage (IVC) systems have been developed that aim to ameliorate these problems. It has been suggested that providing a more stable and protected environment is beneficial to the animals and the personnel working in the animal rooms [11]. Consequently the use of IVC systems has proliferated. However, the design of these systems – although sharing similar features – can vary markedly in a number of ways (e.g. ventilation rates, internal air pressure, and location of air delivery), and so it cannot be assumed that different IVC systems will influence laboratory animals in the same way. Yet, although there is so much variation between different IVC systems, little is known as to what impact these design differences can have on the animals and the data that is generated from them.

Forced ventilation, noise and vibrations could potentially constitute chronic stressors for animals housed within IVC systems – and are often used to induce major changes in behaviour and neurobiology [12–16] – and these are among the factors that typically vary between IVC systems. Previous studies have demonstrated that, when given the choice, mice avoided high ventilation rates and preferred air delivery in the cover of the cage rather than at the level of the animal [10], although see [17], indicating a potential aversion for different factors within the IVC environment. However, when assessing animal welfare – when the aim is to limit an animal's exposure to negative states such as anxiety – we need to consider not only the animal's choice and preference, but also its general behavioural and physiological response to the experience of a particular environment [18–20], what is known as an 'indicator' approach. This is because, whilst it is a valuable technique, preference/choice can be influenced by many factors, including: previous experience [21]; the balance between short and long-term preferences [22]; the influence of stress and affective state [23]; and animals making errors [24].

An indicator approach has previously been used to compare the impact of different IVC systems in comparison to conventional cages on anxiety-related behaviour in mice [6,25], with the authors finding that mice housed in IVCs exhibited reduced activity and increased anxiety-related behaviour compared to those housed in conventional cages. However, Kallnik et al. [6] carried out their study on singly housed mice. Given that recommendations (e.g. Directive 2010/63/EU; Guide for the care and use of Laboratory Animals, U.S. National Research Council (Eighth Edition)) are for the group housing of laboratory mice, it is important to determine if a similar impact is found when mice are housed in groups — particularly given that there can be a degree of resilience against stress provided by the presence of conspecifics [26]. It is also important – given the great variation in design features between different IVC systems – to see whether an indicator approach can identify differences between types of IVC housing system, and few studies have investigated this issue. Champy et al. [27] studied the influence of three different IVC systems (M.I.C.E.® (Animal Care System), SealSafe® Plus (Tecniplast) and Innocage® (Innovive Inc.)) on mouse phenotypes, and found that there was little difference between these particular IVC systems on any of the parameters that they recorded, including anxiety-related behaviour.

An important consideration when investigating the impact of different IVC systems on laboratory rodent behaviour is the potential influence of strain. Kallnik et al. [6] found that IVC housing (IVC Classic) influenced mouse behaviour *generally* (i.e. having the same effect on more than one strain), reducing activity and enhancing anxiety-related behaviour, as well as acting in a *strain-specific* manner (i.e. having differential effects on strains), with increased acoustic startle response observed in C3HeB/FeJ, but not C57BL/6J, mice. In contrast, Mineur and Crusio [25] found only strain-specific effects. For this reason we included two different mouse strains within our study in order to reveal, to at least some extent, any differential effect of housing conditions on the behaviour of different strains of laboratory mouse. We used C57BL/6J and BALB/c as strains that are typically used within laboratory settings [28] and also show contrasting levels of anxiety-related behaviour [29].

The aim of this study was therefore to investigate the impact of two different IVC housing systems on the anxiety-related behaviour and welfare of two strains of laboratory mouse using a variety of behavioural and physiological indicators as recommended when defining and implementing protocols for the welfare assessment of laboratory animals [30].

## 2. Material and methods

### 2.1. Subjects and housing

The subjects were 128 juvenile (6–7 weeks of age) female laboratory naïve mice of two commonly used, but behaviourally contrasting [31,32], strains (C57BL/6J and BALB/c) obtained from a single external supplier (Charles River, Calco, Italy). Mice were individually identified (ear tags) as part of normal facility procedure, allowing us to record individual (e.g. injury/wounds) as well as group (e.g. position within the cage) measures. They were housed (random allocation) in groups of four individuals (same strain) in two types of IVC system. The IVCs differed in specific ways (see 'Housing systems'), but were all provisioned with the same bedding material (hard wood shavings) and ad libitum food (Global Diet 2018S, Harlan Italy, S. Pietro al Natisone, Italy) and water. The mice were kept in the same room within a Specific Pathogen-Free animal facility with a regular 12:12 h light/dark cycle (lights on 07:00 a.m.), at a constant room temperature of 22 ± 2 °C, and relative humidity approximately 55 ± 10%. All cages were changed every 14 days after the first week, and inspected daily. At the end of the study all mice were euthanized by exposure to $CO_2$ according to institutional protocol.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the Mario Negri Institute in compliance with national (Decreto Legge nr 116/92, Gazzetta Ufficiale, supplement 40, February 18, 1992; Circolare nr 8, Gazzetta Ufficiale, July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council (Eighth Edition) 2011).

### 2.2. Housing systems

In this design we compared two IVC systems (SealSafe® Plus (Techniplast) and Allentown) that differed in their air supply delivery systems: (System One) air delivery at the cage 'cover' level; (System Two) air delivery at the 'animal' level. The air supply ventilation rate also varied as both systems were operated according to the manufacturer's specification, with 75 ACH for System One (cover-level) and 50 ACH for System Two (animal-level). The two housing systems also differed slightly in size (*System One*: floor space 501 $cm^2$, height 17 cm, height without cover 13.5 cm, height of air delivery from the cage floor 16.5 cm; *System Two*: floor space 530 $cm^2$, height 17.8 cm, height without cover 13.5 cm, height of air delivery from the cage floor 7.2 cm) and factors including the position of the food hopper, with the food hopper for System One at the back of the cage and the food hopper for System Two at the front. This degree of variation between systems meant that, although we could not identify which specific factor (i.e. air supply delivery or air supply rate or both) resulted in any observed differences in mouse anxiety-related behaviour and welfare, this 'systems approach' would allow us to determine if there were any overall differences between the two housing systems, making the results directly transferable and highly relevant to researchers. If any system differences were identified, then these could be investigated in more detail in further studies. We observed eight cages ($n = 8$) for each of the housing systems ($N = 16$), but as two mouse strains were being studied this gave a total of 128 mice ($N = 32$ cages in total).

### 2.3. Experimental protocol

Cages from the two systems were balanced between racks (e.g. the same number from each system on each rack) and all cages were placed in the middle row of the racks in order to take into account any potential influence of rack location and position-within-rack on mouse behaviour. Measurements were collected over a seven-week period, with the first week of the study allowing the mice to acclimatise prior to the start of the six-week experimental phase, although some measures were collected for all 7 weeks. The study was split across 2 weeks (balanced for strain/system), thus reducing the number of animals to be tested at the end of the study.

Behavioural observations took place between 0900 and 1200 h. For those measures that were repeatable (e.g. bodyweight), data were collected twice a week, however, open field and elevated plus-maze tests only took place at the end of the study. This allowed us to identify both short and longer-term responses to the housing systems, and this was also reflected in the choice of behaviour and anxiety-related indicators utilised.

### 2.4. Measures of general behaviour and anxiety-related behaviour

Whilst we selected measures that we believed would reveal underlying differences in behaviour and anxiety (both short and longer-term), we particularly focused on those measures that could be simply, quickly and reliably recorded by staff (e.g. vets, technicians) to maximise the applicability of our results. Our measures therefore focused either on indirect recording of behaviour (e.g. injury scores as a reflection of aggression), 'challenge' tests that took place outside of the home cage (e.g. tests of anxiety-related behaviour) or unambiguous behavioural observations (e.g. location of mice within the cage).

#### 2.4.1. Indirect behavioural and physical measures

These measures included: injury/wound scores [present/absent, in three zones: head zone; middle zone; and tail zone]; barbering (body hair removal) score [present/absent, in three zones: head zone; middle zone; and tail zone]; whisker-trimming (whisker area only) score [present/absent]; bedding pushing (i.e. is the bedding covering the air delivery pipe in System Two or equivalent height in System One) score (score 0 (no bedding covering the air delivery pipe or equivalent height); 1 (bedding partially covering air delivery pipe or equivalent height); 2 (bedding completely covering air delivery pipe or equivalent height); bodyweight (g); water utilisation (ml); food utilisation (g). These observations were made twice a week (Monday and Friday) for every individual in each cage (e.g. injury/wound score) or each cage (e.g. water utilisation). Bodyweight was also recorded at the end of the study prior to euthanasia. Observations were alternated between cages from the two different housing systems.

#### 2.4.2. Within-cage behaviour

Twice a week (0900–1200), the number of mice in the front half of the cage was recorded for every cage in order to give a quick and unambiguous indication of animal location (i.e. possible values ranged from 0 to 4 animals), with a mouse judged as being in one half of the cage when the majority of the animal's body was in a particular half. In the unlikely event of an animal being 50% in each half of the cage, it was counted as being in whichever side its head was positioned. Observations were alternated between cages from the two different housing systems.

#### 2.4.3. Tests of anxiety-related behaviour

Testing (Open Field (OF), Elevated Plus Maze (EPM)) took place at the same time (approx. 1000–1300) in two different rooms from where the animals were housed, under dim illumination provided by a 60 W lamp placed 1 m above the apparatus and pointed towards the ceiling. For both tests, mice were counted as being in a particular zone/location when all four of its legs were positioned within the zone [33], and the apparatus was wiped with 70% ethanol and dried prior to each test. The order in which animals were tested was alternated between cage systems. Two mice from each cage were tested in the OF, and the other two tested in the EPM. An average from the pairs of mice was calculated to provide a cage average for both tests.

The OF was a grey Perspex box (40 × 40 × 40 cm) with the floor divided into 25 (8 × 8 cm) squares. Mice were placed in the same corner as a 'starting point' and their behaviour video-recorded for 5 min. The number of internal (the nine central squares) and external (the sixteen peripheral squares) squares crossed, the time spent in the central area of the open field (the nine central squares), the number of rears, duration of self-grooming, and the number of faecal boli were scored from video by experienced researchers 'blind' to the housing system as measures of general activity and anxiety-related behaviour.

The EPM was made of black Perspex with two open arms (30 × 5 cm) and two closed arms (30 × 5 cm) extending from a central platform (5 × 5 cm) raised 40 cm above the floor. The closed arms had 25 cm walls and the open arms had 0.5 cm raised lips along the edges. At the beginning of each test mice were placed on the central platform facing an open arm and their behaviour video-recorded for 5 min. The number of entries and the time spent in the open and closed arms, the number of rears, the duration of self-grooming, the number of faecal boli excreted were scored from video by experienced researchers 'blind' to the housing system as measures of anxiety-related behaviour.

#### 2.4.4. Faecal corticosterone

Faecal boli (ten per cage, to get at least the 0.05 g faecal matter required for assay) were collected immediately after the tests of anxiety-related behaviour (i.e. at the end of the study). The boli were stored (labelled Eppendorf, frozen −20 °C) before subsequent analysis by enzyme immunoassay (EIA) to determine the levels of faecal corticosterone metabolites (EIA Kit: ADI-Nr 901-097 Enzo Life Sciences) in accordance with the manufacturer's instructions.

### 2.5. Data analysis

This was a between-subjects design, such that mice were only exposed to one of the two treatment groups/housing systems. Because individual mice within a cage were non-independent, we used 'cage' as our experimental unit, with data collected for all four mice within each cage and their data combined to give a cage average. Consequently, our sample size was n = 8 cages per system/strain (N = 32 cages). Data conformed to parametric statistical requirements (e.g. normality of data). For those measures collected on more than one occasion, we used a repeated measures General Linear Model (GLM) with Housing System (System One, System Two) and Strain (C57BL/6J and BALB/c) as between-subjects factors and Time as a within-subjects factor. For those measures (e.g. tests of anxiety-related behaviour) collected at only one time point, we used a two-way ANOVA (Housing System/Strain). The statistical package used was SPSS (version 19). Only statistically significant results are presented in full. If significant interactions were found, then related main factor results are not presented.

## 3. Results

### 3.1. Indirect behaviour and physical measures

#### 3.1.1. Bodyweight

There was no significant difference between housing systems ($F_{1,28} = 0.411$, $P = 0.527$). There was a statistically significant Strain * Time interaction ($F_{14,392} = 9.8$, $P < 0.001$) which revealed that for days 0, 4 and 7 the C57BL/6J mice were initially heavier than the BALB/c mice, but that this changed over time, with the BALB/c mice becoming heavier than the C57BL/6J mice for days 18, 21 and 25 during the mid-point of the study, but no difference between strains by the end of the study. The growth rate of both mouse strains

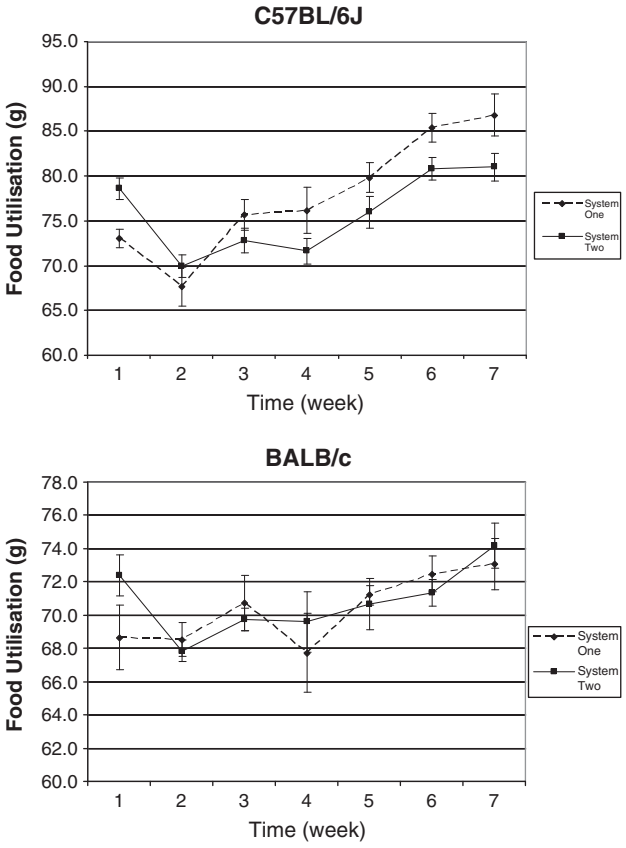


**Fig. 1.** Food utilisation (g) over time (weeks) for System One and System Two, for each mouse strain separately. Data are means ± standard error.

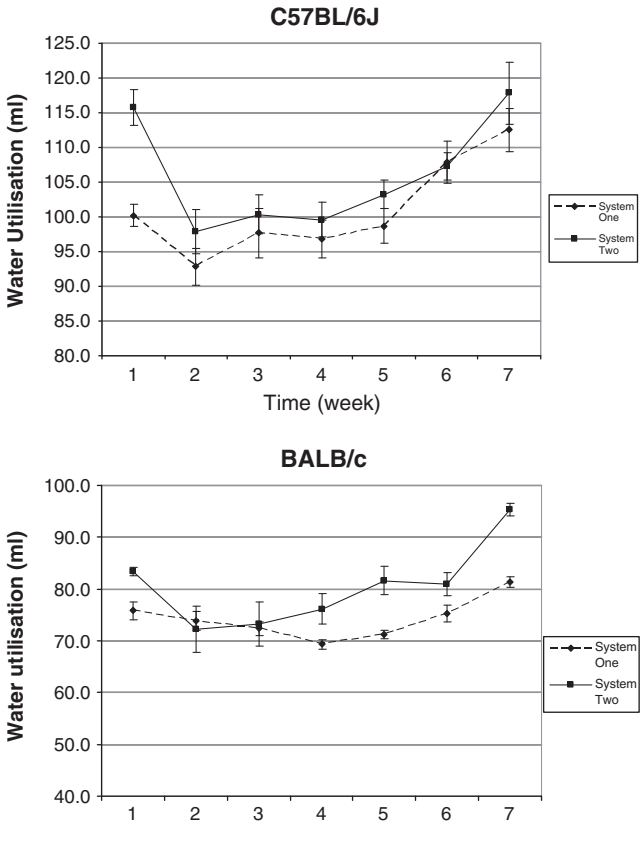


**Fig. 2.** Water utilisation (ml) over time (weeks) for System One and System Two, for each mouse strain separately. Data are means ± standard error.

was comparable to figures provided by the supplier (Charles River) (data not shown).

*3.1.2. Food utilisation*

It should be noted that 'food utilisation' was calculated as change in total food weight (g) and therefore incorporates food removed/lost from the hopper but not necessarily ingested. There was a Time * System interaction ($F_{6,168} = 4.7$, $P < 0.001$) showing that in week 1, mice, regardless of strain, utilised more food when housed in System Two compared to System One (see Fig. 1). There was also a significant Time * Strain interaction ($F_{6,168} = 8.9$, $P < 0.001$) revealing that for weeks 1 and 3–7 C57BL/6J mice utilised more food than the BALB/c mice.

*3.1.3. Water utilisation*

It should be noted that 'water utilisation' was calculated as the change in total water volume (ml) and therefore incorporates water removed/lost (e.g. via evaporation) from the water bottle but not necessarily ingested. There was a System effect ($F_{1,28} = 10.9$, $P = 0.03$), indicating that, regardless of strain and time, mice utilised more water in System Two compared to System One (see Fig. 2). There was also a Strain effect ($F_{1,28} = 143.1$, $P < 0.001$), with C57BL/6J mice utilising more water than the BALB/c mice. Finally, there was an effect of Time ($F_{1,28} = 36.7$, $P < 0.001$), showing that the mice (regardless of strain and housing type) utilised water significantly more in week 1, then this dropped to a lower level before gradually increasing over time. There were no significant interaction effects (System * Strain: $F_{1,28} = 0.03$, $P = 0.865$; System * Time: $F_{6,168} = 0.753$, $P = 0.608$; Strain * Time: $F_{6,168} = 0.785$, $P = 0.583$; System * Strain * Time: $F_{6,168} = 1.49$, $P = 0.184$). In order to identify any potential differences between housing system in their background water evaporation rate (e.g. due to differences in ACH), we compared water loss in four cages of both housing system over a period of 1 week in the absence of mice. We found no difference in water loss ($F_{1,6} = 0.844$, $P = 0.394$) between the housing systems.

*3.1.4. Injury/wound scores*

No injuries/wounds were recorded for any individual mouse of either strain/housing system.

*3.1.5. Bedding pushing score*

There was a System effect ($F_{1,28} = 5.3$, $P = 0.029$), with mice, regardless of strain or time, having higher bedding pushing scores when housed in System Two compared to System One. There was a Time effect ($F_{13,364} = 4.2$, $P < 0.001$), with a general increase in bedding pushing score over time. There was also a Strain effect ($F_{1,28} = 20.3$, $P < 0.001$), with BALB/c mice having higher bedding pushing scores than C57BL/6J mice (see Fig. 3). There were no significant interactions (System * Strain: $F_{1,28} = 0.012$, $P = 0.913$; System * Time: $F_{13,364} = 1.298$, $P = 0.211$; Strain * Time: $F_{13,364} = 1.29$, $P = 0.216$; System * Strain * Time: $F_{6,168} = 0.439$, $P = 0.955$).

*3.1.6. Barbering*

Because barbering, once observed in an individual mouse, continued for the remainder of the study, data were only analysed for the final week of the experiment. There was no System effect ($F_{1,28} = 1.201$, $P = 0.282$). There was a Strain effect ($F_{1,28} = 4.8$, $P = 0.037$), with C57BL/6J mice showing higher scores for barbering compared to BALB/c mice.

*3.1.7. Whisker trimming*

As for barbering, whisker trimming was only analysed for the final week of the experiment. We found no statistically significant

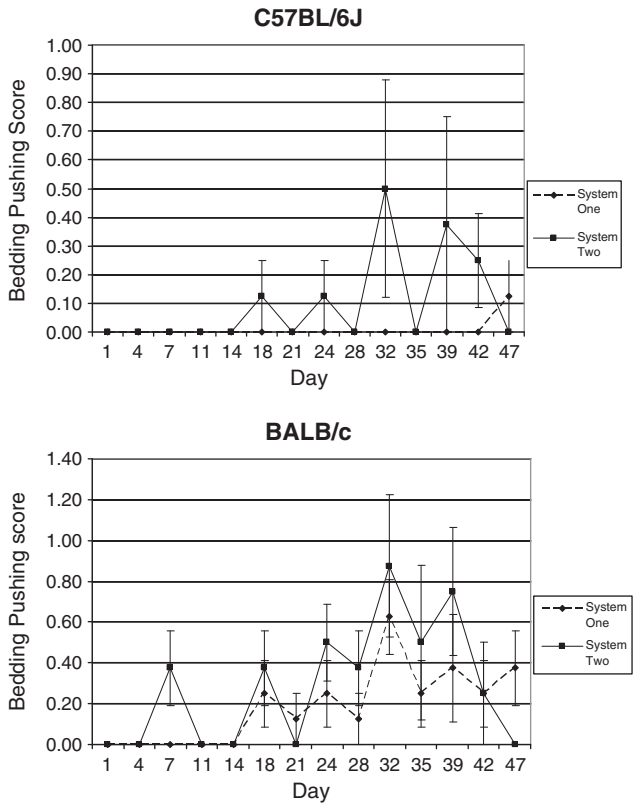


**Fig. 3.** Bedding pushing scores over time (weeks) for System One and System Two, for each mouse strain separately. Data are means ± standard error.

differences either between Systems ($F_{1,28} = 1.697$, $P = 0.203$) or Strain ($F_{1,28} = 0.424$, $P = 0.52$).

### 3.2. Within-cage behaviour

#### 3.2.1. Position of mice in the cage

There was a Strain * System interaction ($F_{1,28} = 8.6$, $P = 0.007$), with both BALB/c and C57BL/6J mice having greater numbers of individuals in the front half of the cage when housed in System Two compared to the System One. There was a Time * System interaction ($F_{13,364} = 2.3$, $P = 0.006$), with all days except days 39 and 42 showing significantly more individuals in the front half of the cage for those mice housed in System Two compared to System One.

#### 3.2.2. Open field

There was a strain effect ($F_{1,28} = 60.2$, $P < 0.001$), with BALB/c mice producing more droppings than C57BL/6J mice. For 'internal' crossing frequency (INT to INT and EXT to INT) there was a Strain * System effect ($F_{1,28} = 7.7$, $P = 0.01$), with C57BL/6J mice showing higher levels of internal crossing when housed in System One compared to System Two, and BALB/c mice showing no difference between housing systems. For 'external' crossing frequency (EXT to EXT and INT to EXT) there was a Strain effect ($F_{1,28} = 76$, $P < 0.001$), with C57BL/6J mice crossing more than BALB/c mice.

Regardless of strain, mice housed in System One spent more time in the internal/central zone than those housed in System Two ($F_{1,28} = 6.2$, $P = 0.019$). C57BL/6J mice also spent more time in the internal/central zone than BALB/c mice ($F_{1,28} = 4.9$, $P = 0.035$) (see Fig. 4). C57BL/6J mice were observed to rear more often than BALB/c mice ($F_{1,28} = 39.6$, $P < 0.001$) and for longer ($F_{1,28} = 12.4$, $P = 0.001$). There was a System effect on grooming duration ($F_{1,28} = 8.2$, $P = 0.008$) with mice housed in the System Two spending more time grooming than those

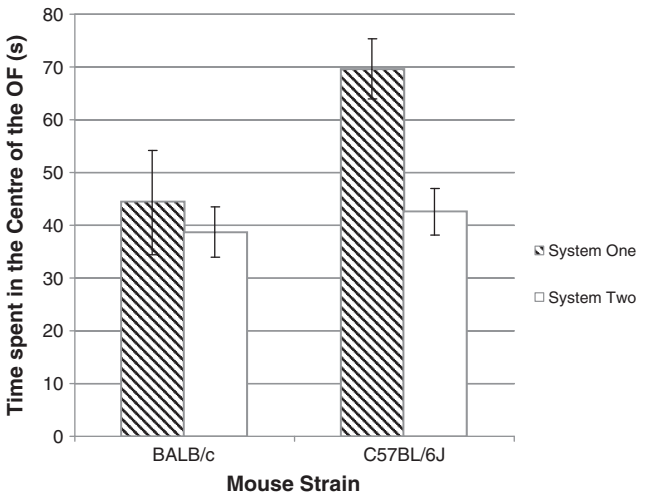


**Fig. 4.** Time spent (s) in the central/internal zone of the OF for System One and System Two, for each mouse strain separately. Data are means ± standard error.

housed in the System One. Although there were no strain differences in the frequency of grooming, C57BL/6J mice spent more time grooming than the BALB/c mice ($F_{1,28} = 10.3$, $P = 0.003$).

#### 3.2.3. Elevated plus maze

There was a System effect ($F_{1,28} = 12.2$, $P = 0.002$), with mice housed in System Two producing more droppings in the EPM than those housed in System One. There was also a strain effect ($F_{1,28} = 19.6$, $P < 0.001$), with BALB/c mice producing more droppings that C57BL/6J mice. We found that mice housed in the System One spent more time in the open arm of the EPM than those housed in System Two ($F_{1,28} = 18.5$, $P < 0.001$) (see Fig. 5). There was also a strain effect, with C57BL/6J mice spending more time in the open ($F_{1,28} = 17.2$, $P < 0.001$) and closed arms ($F_{1,28} = 10.4$, $P = 0.003$) than BALB/c mice, as well as moving more often into the open ($F_{1,28} = 44$, $P < 0.001$) and closed ($F_{1,28} = 29.4$, $P < 0.001$) arms. There was a Systems effect on time spent in the centre of the EPM ($F_{1,28} = 13.6$, $P = 0.001$), with mice housed in System Two spending more time in the centre than those housed in System One. There was also a strain effect, with BALB/c mice spending more time in the centre of the EPM compared to C57BL/6J mice ($F_{1,28} = 34$, $P < 0.001$).

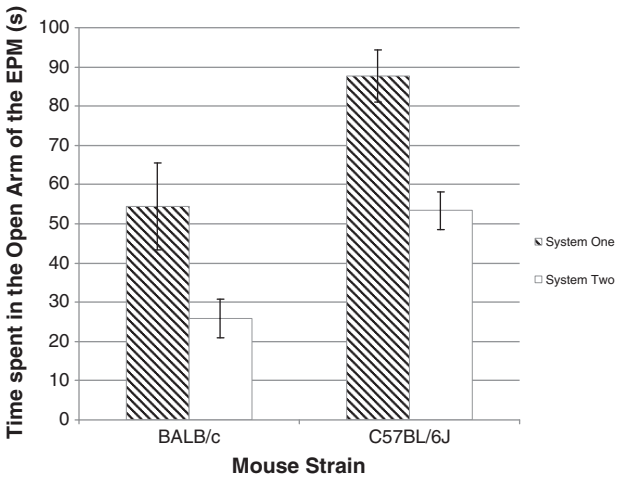


**Fig. 5.** Time spent (s) in the open arm of the EPM for System One and System Two, for each mouse strain separately. Data are means ± standard error.

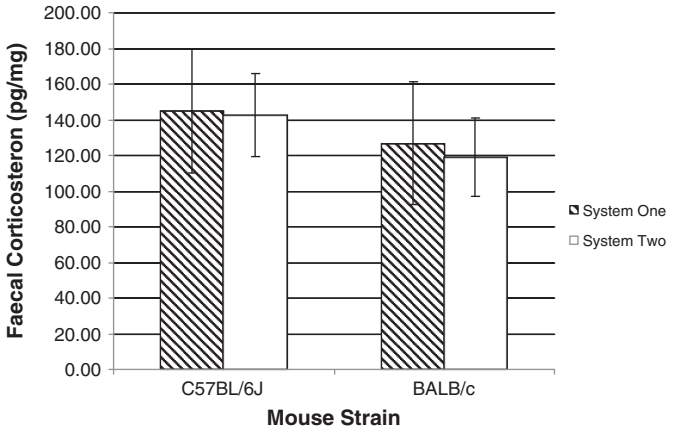


**Fig. 6.** Faecal corticosterone metabolite levels (pg/mg) for System One and System Two for each mouse strain separately. Data are means ± standard error.

### 3.3. Faecal corticosterone

We found no significant differences in the level of faecal corticosterone metabolites between the housing systems ($F_{1,43} = 0.034$, $P = 0.854$), strains ($F_{1,43} = 0.538$, $P = 0.467$), or any interaction ($F_{1,43} = 0.009$, $P = 0.926$) (see Fig. 6).

## 4. Discussion

From the results it appeared that IVC housing System Two (with air delivery at the 'animal' level at 50 ACH) was more anxiety-inducing than IVC housing System One, with air delivery at the 'cover' level (and at 75 ACH), for mice of both strains (BALB/c, C57BL/6J). This was based upon observed differences in anxiety-related behaviour shown by both strains of mouse housed in System Two compared to those housed in System One. In particular, the increase in defaecation in the EPM, the decreased time spent in the open arm of the EPM, and the increased time spent in the starting position in the EPM for mice housed in System Two. Similar results were found in the OF test, with mice housed in System Two spending less time in the central/internal zone of the arena, suggesting reduced confidence compared to those mice housed in System One.

These findings reflect those of Kallnik et al. [6] and Mineur and Crusio [25] who observed reduced activity and enhanced anxiety-related behaviour in mice housed in IVC systems compared to 'conventional' housing, and extends this finding to group housed mice when housed in two different types of IVC housing system. Thus, not only does it appear that some IVC housing systems can increase anxiety, as determined by anxiety-related behaviour, compared to conventional systems [6,25], but there also appear to be significant differences between IVC systems in their influence on mouse behaviour and anxiety; including when group-housed. In contrast to our findings, however, Champy et al. [27] found little effect of the three different IVC systems that they compared on any of the parameters that they recorded — including anxiety-related behaviour in the open field test. The three IVC systems that they compared were IVC M.I.C.E.® (Animal Care System), IVC SealSafe®Plus (Tecniplast), and Innocage® (Innovive Inc.). These particular IVC systems differ in a great number of features, including shape of cage, and construction material, as well as aspects of ventilation (e.g. air flow rate and pressure). It is therefore perhaps surprising that they did not appear to observe any difference in anxiety-related behaviour between the systems. However, one possibility is that because their mice were anxiety tested after 2 weeks of acclimatisation, this may not have been sufficient time to induce differential levels of emotionality as compared to the 7 weeks prior to the testing of anxiety-related behaviour in our study. Although changes to the housing environment can result in immediate behavioural changes, influences on affective state may take longer to establish. For example, changes in anxiety-like behaviour in mice were observed after 6 weeks of environmental enrichment [34].

When assessing animal preference in IVC systems, Baumans et al. [10] found that mice avoided high ventilation rates and preferred air supply access in the cover of the cage rather than at the level of the animal, demonstrating that mice actively choose between IVC housing systems that vary in particular features. Our results indicate that IVC systems that vary in these same features also appear to influence anxiety-related behaviour (in the absence of choice). Taken together, the findings from these two approaches suggest a reduction in welfare in mice housed in animal level ventilation systems. Interestingly, if, as determined by Baumans et al. [10], mice prefer air entry from the 'cover' level and low ventilation rates, then our data suggest that air entry level may be a significant feature in mouse choice, given that our mice exhibited least anxiety-related behaviour when housed in cages with air entry at the cover level (preferred) despite also having higher ventilation rates (avoided). This appears to reflect the findings of Krohn and Hansen [17] who found that it was the presence of draughts that influenced mouse choice rather than the number of air changes per se. Clearly, further research is needed to disentangle these features.

Grooming within the OF can be considered a sign of anxiety-related behaviour (e.g. as a displacement activity) and this was higher in the mice housed in System Two. However, although the strain effects observed in the OF typically followed the general pattern of Balb/c mice exhibiting more anxiety-related behaviour than C57BL/6j mice (see later), grooming behaviour was an exception to this, with C57BL/6j mice grooming for longer than Balb/c mice. The interpretation of grooming behaviour can vary, because it increases in the contrasting contexts of both stress and comfort, as well as between strains [35]. Consequently, data relating to position within the OF (and EPM) may be more reliable indicators of putative anxiety level.

Strain differences mainly reflected the increased anxiety-like behaviour of BALB/c compared to C57BL/6J mice, with BALB/c mice showing increased defecation in both OF and EPM, less time in the open arms of the EPM and less time in the internal/central zone of the OF. We also observed that BALB/c mice were reluctant to leave the starting point of the EPM. These results reflect the general finding in the research literature that BALB/c mice show more anxiety-like behaviour than C57BL/6J mice [29,36]. We did, however, observe higher levels of barbering in the C57BL/6J mice, which reflects epidemiological research revealing that C57BL/6J mice were likely to exhibit barbering behaviour [37] — suggesting a potential dissociation between barbering behaviour and putative anxiety level.

The confirmation in our own study of the predicted differences in strain-related emotionality thus strengthens our results concerning the observed anxiety-related differences between the housing systems. Although the majority of results followed a pattern of independent effects of both strain and housing system, we did also observe some interactions between these factors (e.g. internal crossing in the OF). Thus, along with Kallnik et al. [6], but in contrast to Mineur and Crusio [25], we found both general and strain-specific effects of IVC housing system on mouse anxiety-like behaviour. In addition, it is perhaps worth emphasising the importance of selecting the appropriate strain when investigating the impact of the housing environment. In order to show a behavioural change in response to a particular housing environment, animals need to be at a level that allows for potential change. For example, BALB/c mice may not have shown the same reduction in frequency of 'internal crossing' in the OF as C57BL/6J mice when housed in System Two, because they were already at a low level of activity and could therefore not go any lower.

Mouse position within a cage can be influenced by a variety of factors including activity levels, food hopper position, the level of light/disturbance and/or refuge location [38]. Behaviour within the cages

appeared to back up the findings from the tests of anxiety-related behaviour, that System Two resulted in increased anxiety-like behaviour, because mice housed in System Two were more frequently found in the front half of the cage — away from the position of air entry. This could suggest that the mice in System Two were avoiding the area of the cage where air entered – despite this resulting in them having to spend more time in the front of the cage – an observation that would reflect mouse avoidance of aversive stimuli/environments [39]. However, position in the cage could also have been influenced by differences in cage design between the two housing systems (e.g. location of food hopper) and so has to be interpreted with caution. In System One the food hopper was located at the back of the cage, whilst for System Two the food hopper was at the front of the cage — the opposite end to the air delivery. This could explain the observed increase in time spent at the front of the cage in System Two — although our observations were in the light period when mice would typically be inactive rather than feeding.

Mice were also found to have higher 'bedding pushing' scores in System Two, a finding that may have reflected attempts to cover the point of air entry — a potential response to what may be considered an aversive stimulus as seen in the 'defensive burying' paradigm [40]. Mice in IVC systems have previously been observed to build higher walled nests that may act to protect them against draughts [10]. Evidence that this behaviour might be related to an anxiety-like state is suggested by the fact that we also observed a strain effect on 'bedding pushing', with BALB/c mice doing more bedding pushing than C57BL/6J mice in both IVC systems, potentially reflecting commonly found differences in anxiety-like behaviour between these two strains [29].

There was also a potentially interesting result in that mice housed in System Two utilised more water (as measured by water loss) than those housed in System One. This result could be explained by differences in ventilation rate between the two housing systems (System One: 75 ACH; System Two: 50 ACH). However, as we found no differences in evaporation rate between the two Systems when they did not contain mice, this result suggests that the difference in water loss was due to the activity of the mice — although consumption may not be the only factor involved. Other authors have interpreted similar increases in apparent drinking behaviour as indicating prolonged stress, i.e. polydipsia [41,42], which would reflect the higher levels of anxiety-related behaviour that we observed for mice housed in System Two.

We observed an unexpected difference between housing systems in food utilisation in the first week of the study. The overall high level of food utilisation during the first week (compared to week 2) after arrival is typical for the period of initial acclimatisation to the laboratory environment following the stress of transport [43]. However, it is not immediately clear why there might have been a difference during this period between the two systems — one possibility being that the previously discussed differences in food hopper location/food acquisition may have required more familiarisation due to initial novelty for those animals in System One (that fed less).

Perhaps surprisingly, we did not observe a difference in the level of faecal corticosterone metabolites for those mice housed in System Two compared to System One. Other studies have demonstrated significant changes in faecal corticosterone metabolite levels as a consequence of changes to the housing environment (e.g. from 14 to 70 days after environmental enrichment: [44]; following removal of individuals: [18]; following single housing: [45]). However, Gurfein et al. [44] also assessed the impact of environmental enrichment on anxiety behaviour in the EPM, but found no significant effects — thereby demonstrating that expected correlations between different measures of stress and welfare may not always be revealed [46].

In conclusion, it appears that for the two strains of laboratory mice observed in this study, being housed in System Two (air entry at the 'animal' level at 50 ACH) resulted in more behavioural indicators of anxiety than being housed in System One (air entry at the 'cover' level at 75 ACH). This provides further evidence that changes in the housing environment – even between two different types of IVC housing system – have the potential to impact upon anxiety-like behaviour in mice and, as a consequence, the robustness and comparability of experimental data. This reflects the importance of taking such potential influences into consideration during experimental design and when interpreting and comparing results. Specifically, it demonstrates that the term 'IVC' cannot be generalised across different IVC systems, but that variation between these systems may well have differential influences upon mouse behaviour and research data.

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